APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2006, p. 4805–4810 Vol. 72, No. 7
0099-2240/06/$08.00+0 doi:10.1128/AEM.00416-06

Asymmetrical Interactions between *Wolbachia* and *Spiroplasma* Endosymbionts Coexisting in the Same Insect Host

Shunsuke Goto,[1,2] Hisashi Anbutsu,[1] and Takema Fukatsu[1,2]*

Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566,[1] and Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572,[2] Japan

Received 20 February 2006/Accepted 8 May 2006

We investigated the interactions between the endosymbionts *Wolbachia pipientis* strain *w*Mel and *Spiroplasma* sp. strain NSRO coinfecting the host insect *Drosophila melanogaster*. By making use of antibiotic therapy, temperature stress, and hemolymph microinjection, we established the following strains in the same host genetic background: the SW strain, infected with both *Spiroplasma* and *Wolbachia*; the S strain, infected with *Spiroplasma* only; and the W strain, infected with *Wolbachia* only. The infection dynamics of the symbionts in these strains were monitored by quantitative PCR during host development. The infection densities of *Spiroplasma* exhibited no significant differences between the SW and S strains throughout the developmental course. In contrast, the infection densities of *Wolbachia* were significantly lower in the SW strain than in the W strain at the pupal and young adult stages. These results indicated that the interactions between the coinfecting symbionts were asymmetrical, i.e., *Spiroplasma* organisms negatively affected the population of *Wolbachia* organisms, while *Wolbachia* organisms did not influence the population of *Spiroplasma* organisms. In the host body, the symbionts exhibited their own tissue tropisms: among the tissues examined, *Spiroplasma* was the most abundant in the ovaries, while *Wolbachia* showed the highest density in Malpighian tubules. Strikingly, basically no *Wolbachia* organisms were detected in hemolymph, the principal location of *Spiroplasma*. These results suggest that different host tissues act as distinct microhabitats for the symbionts and that the lytic process in host metamorphosis might be involved in the asymmetrical interactions between the coinfecting symbionts.

Endosymbiotic microorganisms are commonly found in a diverse array of insects (3, 6). Some symbionts are of a mutualistic nature and contribute to the fitness of their hosts (3), while other symbionts are rather parasitic and tend to affect their hosts negatively (36). In general, these symbionts are maintained through host generations by vertical transmission from mothers to their offspring, often causing considerable effects on various biological aspects of the host insects (3, 36).

Interestingly, some symbionts, including *Wolbachia*, *Spiroplasma*, *Rickettsia*, *Arsenophonus*, *Cardinium*, and others, have evolved the ability to cause reproductive alterations in their arthropod hosts, such as cytoplasmic incompatibility (CI), parthenogenesis, feminization, and male killing. Since these symbionts cannot survive outside the host cells and tissues, it is crucial for them to be vertically transmitted through the host matrilines. Hence, the reproductive alterations effectively increase the frequencies of infected females in the host populations, often at the expense of host fitness. By causing such reproductive manipulations in a selfish manner, these maternally inherited symbionts are able to rapidly spread their infection in the host population (3, 36, 45, 50).

The infection density of symbionts is among the most important factors for understanding their biological effects because it can affect various aspects of endosymbiosis, such as the intensity of reproductive phenotypes, the level of fitness effects, the fidelity of vertical transmission, and others (1, 9, 20, 23, 29, 32, 33, 35, 40, 49). A reduced infection density may result in imperfect vertical transmission and attenuated phenotypic effects, which can lead to a loss of infection in host populations. An excessive infection density may lead to enhanced phenotypic effects on the host, causing pathology at an extreme, which can significantly influence host fitness. On account of the selective pressures acting on both partners, it is expected that some mechanisms should have evolved to control infection density within an appropriate range, which must be governed by the symbiont genotype as well as the host genotype (27, 31, 32).

In addition, symbiont-symbiont interactions may substantially affect infection densities. Coinfections with several different strains or species of symbionts in the same host are commonly found in diverse insect groups (6, 11, 12, 22, 41, 46, 47). The state of being confined in the same host body must facilitate various interactions between the coexisting symbionts. The symbionts may compete for available resources and space in the host body, or they may share the resources and habitats by regulating their own exploitation so as not to damage the whole symbiotic system (23, 43). Considering that different tissues in the same host body must constitute distinct microenvironments for symbionts, an insect body consists of heterogeneous microhabitats for symbionts. Some tissues may be nutritionally favorable, immunotolerant, and/or easy to colonize, whereas other tissues may be nutritionally poor, immunologically hostile, and/or difficult to utilize. If so, it is expected that the coexisting symbionts may colonize, proliferate, and behave in different tissues in different ways and that the local-

* Corresponding author. Mailing address: National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 6, Tsukuba 305-8566, Japan. Phone: 81-29-861-6087. Fax: 81-29-861-6080. E-mail: t-fukatsu@aist.go.jp.

ization and density of one partner might be affected by those of the other. However, such ecological aspects of coinfecting symbionts have been poorly investigated, mainly because of technical difficulties associated with the fastidious nature of symbiotic microorganisms. It is only recently that quantitative PCR approaches have shed light on such microbial interactions in the endosymbiotic system inside an insect body (20, 23, 27, 31, 32, 43).

Thus far, *Wolbachia* and *Spiroplasma* endosymbionts have been identified from natural and laboratory populations of the fruit fly *Drosophila melanogaster* (7, 16, 17, 30, 44, 51). In the host insect, *Wolbachia*, a member of the α subdivision of the class *Proteobacteria*, has been known to induce weak CI (4, 16, 44), while *Spiroplasma*, a member of the class *Mollicutes*, has been reported to cause strong male killing (1, 30, 37). It was of great interest to determine which interactions would be observed between these phylogenetically and phenotypically distinct reproductive manipulators coexisting in the same host body.

In this study, we investigated the interactions between *Wolbachia* and *Spiroplasma* coinfecting *D. melanogaster*. By making use of antibiotic therapy, temperature stress, and hemolymph microinjection, we successfully generated a doubly infected and two singly infected fly strains of all possible infection types in the same host genetic background. By use of these strains, the infection densities of the symbionts were monitored and compared during host development. Interestingly, we identified stage-specific and asymmetrical interactions between the coinfecting symbionts: in pupae and young adults, *Spiroplasma* organisms negatively affected the population of *Wolbachia* organisms, while *Wolbachia* organisms did not influence the population of *Spiroplasma* organisms.

MATERIALS AND METHODS

Original flies and symbionts. The original fly strain, which is called SW in this study, was a wild-type Oregon-R stock of *D. melanogaster* harboring the male-killing *Spiroplasma* sp. strain NSRO. In the 1960s, the Oregon-R fly, which had initially been free of *Spiroplasma*, was injected with hemolymph of the tropical fruit fly *Drosophila nebulosa* carrying NSRO. The transfected flies stably maintained and inherited the symbiont infection, expressed an almost complete male-killing phenotype, and have been reared in the laboratory for over 40 years (1, 38). Our recent inspection of the strain by diagnostic PCR revealed that the fly stock was actually doubly infected with *Spiroplasma* and *Wolbachia*. The nucleotide sequence of the *wsp* gene from the *Wolbachia* sp. (588 bp) was completely identical to the sequences from *Wolbachia pipientis* strain *w*Mel and allied strains that had commonly been identified in *D. melanogaster* (39, 52). Thus, the *Wolbachia* strain used in this study is no doubt genetically close to strain *w*Mel, although the so-called *w*Mel strain may contain considerable genetic variation (39). The flies were reared with a standard cornmeal medium in plastic vials at 25°C by using a long-day regimen (16 h of light-8 h of dark), unless otherwise described. Since *Spiroplasma*-infected flies produce all-female broods, males were supplied from an uninfected Oregon-R strain for maintenance of the fly stock.

Establishment of fly strains. The following strains of *D. melanogaster*, which have essentially the same genetic background and differ only in their symbiont infections, were established: the original SW strain, doubly infected with *Spiroplasma* and *Wolbachia*; the S strain, infected with *Spiroplasma* only; the W strain, infected with *Wolbachia* only; and the U strain, with no symbiont infection.

It is known that *Spiroplasma* infection is sensitive to temperature, optimally maintained at about 23 to 26°C, and unstable at lower or higher temperatures (T. Koana, personal communication). We maintained the SW strain at 18°C for three successive generations. The insects were then individually reared under standard conditions (25°C) to establish isofemale lines and subjected to diagnostic PCR to detect symbiont infection, whereby an isofemale line infected with *Wolbachia* only was established as the W strain.

The SW strain was reared with cornmeal medium containing 2 mg/ml of tetracycline for a generation, and the offspring were individually maintained with normal medium without the antibiotic. The isofemale lines were subjected to diagnostic PCR to detect symbiont infection, by which an isofemale line without symbiont infection was selected as the U strain.

The hemolymph from adult females of the SW strain was injected into adult females of the U strain as described previously (1). The offspring were individually maintained to establish isofemale lines and subjected to diagnostic PCR to detect symbiont infection, by which an isofemale line infected with *Spiroplasma* only was selected as the S strain.

Sampling of insects. To collect insect samples of the SW, W, and S strains at different developmental stages, 10 adult females (1 week after emergence) and 5 adult males (within 5 days after emergence) were reared in plastic vials with cornmeal medium. Eggs and first-, second-, and third-instar larvae were harvested 12, 36, 84, and 108 h after the start of oviposition, respectively. Early and late pupae were collected 156 and 204 h after the start of oviposition. Adult insects were collected within 12 h of emergence and 1, 2, 3, 4, and 5 weeks after emergence. The collected samples were immediately preserved in acetone until DNA extraction (10).

Tissue preparation. Adult females were collected within 24 h of emergence, reared for a week, and subjected to dissection of their ovaries, Malpighian tubules, and guts. Individual insects were carefully dissected with fine forceps under a dissecting microscope in a petri dish filled with phosphate-buffered saline containing Tween 20 (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 175 mM NaCl, 0.1% Tween 20 [pH 7.4]). Isolated tissues were individually washed several times with fresh phosphate-buffered saline containing Tween 20 to minimize possible microbial contamination and were immediately subjected to DNA extraction. Hemolymph samples were collected from adult females by using thin glass capillary tubes as described previously (2).

DNA extraction. The insect samples preserved in acetone were briefly air dried and subjected to DNA extraction by using a QIAamp DNA mini kit (QIAGEN). The purified DNA from a sample was eluted with 200 μl of AE buffer supplied in the kit. Samples of adults, pupae, and second- and third-instar larvae were individually subjected to the DNA extraction procedure. Because eggs and first-instar larvae were too small to be treated individually, 100 individuals were combined and subjected to the DNA extraction procedure. Hence, the samples of eggs for all the strains and of first-instar larvae for the W strain contained both females and males, while the samples of adults, pupae, and second- and third-instar larvae contained all females. The tissue samples were individually subjected to DNA extraction by using a NucleoSpin tissue kit (Macherey-Nagel). The purified DNA from a sample was eluted with 100 μl of BE buffer supplied in the kit.

Diagnostic PCR. PCR detection of *Spiroplasma* and *Wolbachia* was performed by using the primers 16SA1 and TKSSsp for the 16S rRNA gene of *Spiroplasma* (13) and the primers wsp81F and wsp691R for the *wsp* gene of *Wolbachia* (52). The PCR temperature profile was 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min.

For sexing of the W strain at the larval and pupal stages, a PCR targeting *kl5*, a Y chromosome gene of *D. melanogaster* (14), was performed by using the primers kl5-1 and kl5-2 (1). The PCR temperature profile was 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min.

Quantitative PCR. Infection densities of *Spiroplasma* and *Wolbachia* were measured by a quantitative PCR technique in terms of *dnaA* and *wsp* gene copies per elongation factor 1α (*ef1*α) gene copy from the host insect, respectively. Note that the symbiont densities were not estimated by direct cell counts but by gene copy numbers. Quantitative PCR was performed by using TaqMan PCR and an ABI Prism 7700 sequence detection system (PE Applied Biosystems) essentially as described previously (1, 2, 26). The *dnaA* gene of *Spiroplasma* was quantified by using the probe DnaA180T (5′-AGCTTCAAATCCACCAAGATCATCA GGA-3′) and the primers DnaA109F (5′-TTAAGAGCAGTTTCAAAATCGG G-3′) and DnaA246R (5′-TGAAAAAAACAAACAAATTGTTATTACTTC-3′). The *wsp* gene of *Wolbachia* was quantified by using the probe Wsp422T (5′-TT GGTGTTGGTGTTGGTGCAGC-3′) and the primers Wsp355F (5′-GCAATT TCAGGATTAGTGAACGTG-3′) and Wsp477R (5′-ATTCACAGCGGGTTC CAAAG-3′). The *ef1*α gene of *D. melanogaster* was quantified by using the probe EF157T (5′-CAAGTCGACGACCACCGGCCAC-3′) and the primers EF23F (5′-TTAACATTGTGGTCATTGGCCA-3′) and EF123R (5′-CTTCTCAATCG TACGCTTGTCG-3′). The reaction mixture was composed of 1× TaqMan PCR buffer, 4 mM $MgCl_2$, 0.2 mM (each) of dATP, dGTP, dCTP, and dUTP, 0.2 μM (each) of forward primer, reverse primer, and fluorescence-labeled probe, 0.02 U/μl AmpliTaq Gold DNA polymerase, and template DNA. In each 0.2-ml plastic optical tube, 21 μl of master reaction mixture and 4 μl of sample DNA solution were combined and subjected to quantification using an ABI Prism 7700

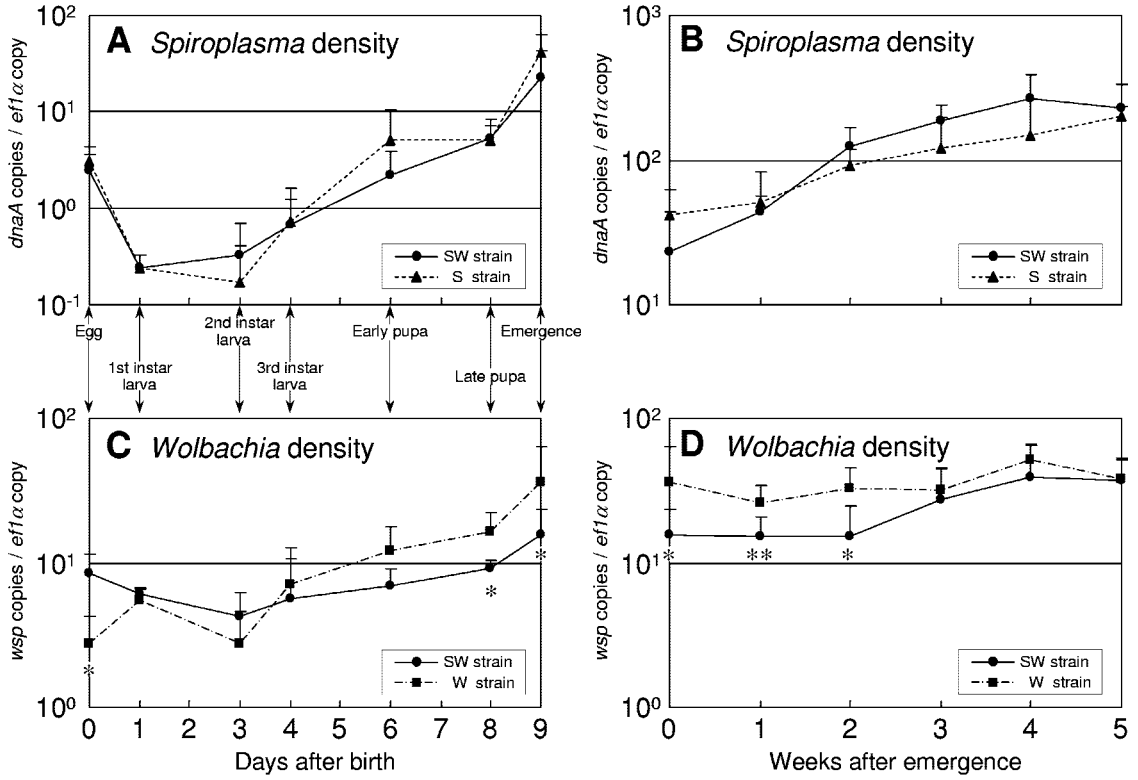

FIG. 1. Density dynamics of *Spiroplasma* and *Wolbachia* during host development in doubly infected and singly infected strains of *D. melanogaster*. The graphs show infection densities of *Spiroplasma* in the egg, larval, and pupal stages (A) and during host adult aging (B) and infection densities of *Wolbachia* in the egg, larval, and pupal stages (C) and during host adult aging (D). Symbols and error bars show means and standard deviations, respectively. Asterisks indicate statistically significant differences (Mann-Whitney U test after Bonferroni correction) (*, $P < 0.05$; **, $P < 0.01$). Sample sizes were as follows: $n = 5$ for eggs and first-instar larvae; $n = 10$ to 12 for second- and third-instar larvae, early and late pupae, and 0-, 1-, and 2-week-old adults; $n = 7$ or 8 for 3- and 4-week-old adults; and $n = 5$ for 5-week-old adults. Note that 100 individuals for each sample were collectively measured without sexing for the egg and first-instar stages, while individual female insects were measured at the later stages.

sequence detector with a temperature profile of 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 55°C for 1 min. Standard curves were drawn by using standard PCR product samples that contained the respective genes at concentrations of 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 copies/μl.

RESULTS AND DISCUSSION

Coexisting *Wolbachia* symbiont does not affect infection density of *Spiroplasma* during host development. Figure 1A shows the density dynamics of *Spiroplasma* during host development from egg to adult emergence in the SW and S strains. From the first instar to adult emergence, the infection density of *Spiroplasma* steadily increased as the host development proceeded. From egg to first instar, the bacterial density dropped, which was probably caused by an arrest of host cell division (suppressed *ef1*α gene copy number) in half of the eggs due to male killing. Figure 1B shows the density dynamics of *Spiroplasma* during adult aging in the SW and S strains. The infection density of *Spiroplasma* increased steadily as host aging proceeded. At all developmental stages, no significant differences in the density of *Spiroplasma* were detected between the doubly infected and singly infected strains.

Coexisting *Spiroplasma* symbiont negatively affects infection density of *Wolbachia* at the pupal and young adult stages of host development. Figure 1C shows the density dynamics of *Wolbachia* during host development from egg to adult emergence in the SW and W strains. During postembryonic development, the infection density of *Wolbachia* generally increased as the host development proceeded. Significantly lower densities of *Wolbachia* in the doubly infected strain were identified at the pupal and newly emerged adult stages. At the egg stage, the bacterial density was apparently higher in the doubly infected strain, which might be due to male killing in the SW strain. Figure 1D shows the density dynamics of *Wolbachia* during adult aging in the SW and W strains. Significantly lower densities of *Wolbachia* in the doubly infected strain were identified 0, 1, and 2 weeks after adult emergence. These results indicated that the infection density of *Wolbachia* was negatively affected in the presence of coinfecting *Spiroplasma* organisms at the pupal and young adult stages.

Possible mechanism underlying asymmetrical interaction between coinfecting *Wolbachia* and *Spiroplasma*. Interestingly, the interactions between coinfecting *Wolbachia* and *Spiroplasma* were asymmetrical, i.e., *Spiroplasma* negatively affected *Wolbachia*, while *Wolbachia* did not affect *Spiroplasma*. What is the basis of the unidirectional suppression of bacterial populations in the endosymbiotic system? It should be noted that these bacterial symbionts may occupy different microhabitats in the same host body. *Wolbachia* endosymbionts are known to

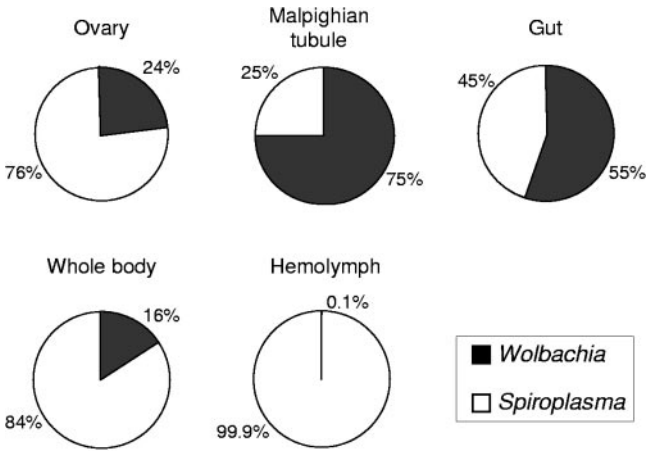

FIG. 2. Relative infection densities of *Spiroplasma* and *Wolbachia* in 1-week-old adult tissues of the doubly infected strain of *D. melanogaster*. Means of bacterial gene copy number data were used to calculate the proportions. Sample sizes were as follows: $n = 4$ for hemolymph and $n = 19$ or 20 for the other tissues. Each of the tissues was prepared from a single female insect.

be endocellular, inhabiting the cytoplasm of a wide array of host cells of both germ and somatic tissues (8, 19, 21). On the other hand, *Spiroplasma* endosymbionts are often found abundantly in the host hemolymph (1, 38). Previous quantitative studies have suggested that although *Spiroplasma* cells are certainly present inside the host cells, their main location is extracellular, in the body cavity (2, 42). Thus, it was conceivable that the different tissue tropisms of the symbionts might be somehow involved in the asymmetrical interactions.

Coexistence and relative densities of *Wolbachia* and *Spiroplasma* in different adult tissues. Figure 2 shows the relative densities of *Wolbachia* and *Spiroplasma* in 1-week-old adult tissues of the doubly infected SW strain. The relative densities were remarkably different among the tissues. *Spiroplasma* was predominant in the ovaries, whereas *Wolbachia* was the majority organism in Malpighian tubules. The level of *Spiroplasma* was comparable to that of *Wolbachia* in the gut. Hemolymph was substantially occupied by *Spiroplasma* only. In the whole body, on the basis of gene copy numbers, the density of *Spiroplasma* was estimated to be about five times higher than that of *Wolbachia*.

Possible involvement of different tissue tropisms of *Wolbachia* and *Spiroplasma*. These results suggest that *Wolbachia* must always coexist and potentially interact with *Spiroplasma* in the intracellular habitat, whereas the majority of *Spiroplasma* organisms are free of such interaction in the extracellular habitat. It appears plausible, although speculative, that the partially overlapping but distinct microhabitats of the symbionts are responsible for the unidirectional suppression of bacterial populations.

Possible involvement of relative abundances of *Wolbachia* and *Spiroplasma*. In addition, the relative abundances of these symbionts might be relevant to their asymmetry. In the whole body of the host, *Spiroplasma* was estimated to be about five times more abundant than *Wolbachia* (Fig. 2). It is conceivable that the minor symbiont *Wolbachia* was severely affected by the coexisting major symbiont *Spiroplasma*, whereas the major symbiont was less influenced by the minor one. Of course, we should be cautious in making such an argument on the basis of quantitative PCR data, considering the possibility that the gene copy numbers may not faithfully reflect the symbiont cell numbers. It is conceivable that the genome copy number per cell of the symbionts may not always be constant, as it is possibly affected by the bacterial growth rate and other environmental factors. Note that the genome copy number in an aphid endosymbiont was reported to be drastically multiplied, and the level of the symbiont genome polyploidy varied throughout host development (24, 25). To date, however, no genome amplification has been reported for *Wolbachia* and *Spiroplasma* endosymbionts. The proliferation rates of the symbionts were not remarkably different between the doubly infected and singly infected strains throughout the host's developmental course (Fig. 1).

Which host tissue is the principal site of *Wolbachia* suppression? Figure 3A shows the infection densities of *Spiroplasma* in 1-week-old adult tissues of the SW and S strains. Although the tissue densities of *Spiroplasma* tended to be higher in the doubly infected strain than in the singly infected strain, the differences

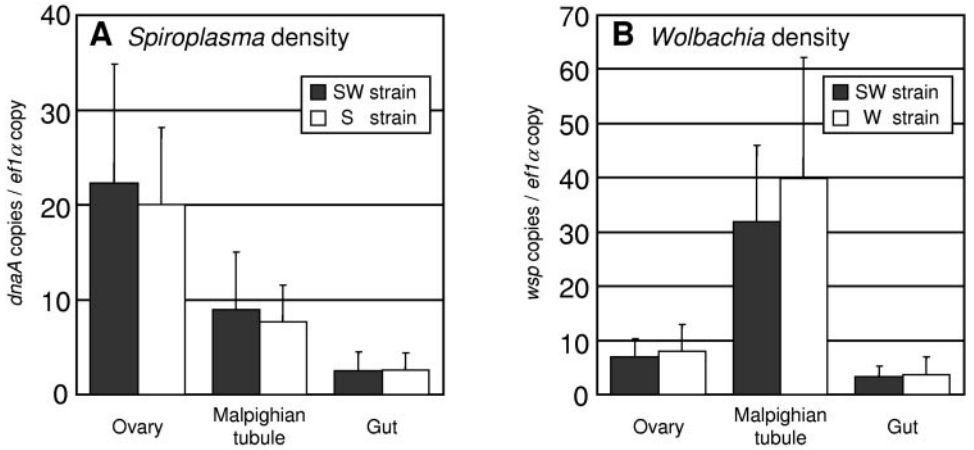

FIG. 3. Infection densities of *Spiroplasma* (A) and *Wolbachia* (B) in 1-week-old adult tissues. Means and standard deviations are shown. The sample size was 19 or 20 for each tissue. Each of the tissues was prepared from a single female insect.

were not statistically significant. Figure 3B shows the infection densities of *Wolbachia* in 1-week-old adult tissues of the SW and W strains. Although the tissue densities of *Wolbachia* tended to be lower in the doubly infected strain than in the singly infected strain, the differences were not statistically significant. These results did not indicate the principal site where the infection density of *Wolbachia* was suppressed by the coexisting *Spiroplasma* organisms. The density of *Wolbachia* might be negatively affected in the whole body of the doubly infected strain, irrespective of the host tissue. Of course, the possibility cannot be excluded that other tissues not examined in this study might comprise the principal site of suppression. One possible site of *Wolbachia* density suppression is the hemolymph, the main location of *Spiroplasma*. However, quantitative PCR analysis revealed that hemolymph samples from the singly infected W strain contained few *Wolbachia* cells (data not shown), which indicated that the hemolymph cannot be the site responsible for the difference. The nature of the interaction between the coexisting symbionts and whether it operates directly between the symbionts or indirectly via the host are also obscure.

Why is the *Wolbachia* density suppressed at pupal and young adult stages of the host? Suppressed infection densities of *Wolbachia* were preferentially observed at the pupal and young adult host stages (Fig. 1C and D), suggesting the possibility that metamorphosis of the host insect may be involved in *Wolbachia*-specific suppression. In holometabolic insects, including *D. melanogaster*, a drastic reorganization of tissues and organs occurs in the process of pupation: the larval tissues disintegrate in apoptotic and degenerative manners, while the adult tissues newly and rapidly develop from imaginal discs and other primordial tissues (15). This situation appears to be more stressful for *Wolbachia*, which is obligately endocellular, than for *Spiroplasma*, which can proliferate extracellularly. In order to not be digested in the apoptotic process, *Wolbachia* organisms infecting the larval tissues may escape from the larval host cells and infect the newly formed adult tissues. On the other hand, since it is mainly extracellular, *Spiroplasma* may be less affected by the apoptotic process. It appears plausible, although speculative, that the lytic process of metamorphosis obscured the boundary of the intracellular and extracellular habitats of the symbionts, forcing the endocellular *Wolbachia* organisms to be transiently extracellular and thus exposed to interaction with extracellular *Spiroplasma* organisms.

Different tissue tropisms of *Wolbachia* and *Spiroplasma*. Quantitative PCR analysis of the dissected tissues revealed that *Spiroplasma* and *Wolbachia* consistently exhibited their own tissue tropisms in the doubly infected and singly infected strains. *Spiroplasma* was the most abundant symbiont in the ovary, and *Wolbachia* showed the highest density in Malpighian tubules, while both symbionts were detected in all tissues examined (Fig. 3). These patterns strongly suggest that host tissues are certainly heterogeneous microhabitats for symbionts and that symbionts colonize, proliferate, and behave in these tissues in different ways. The mechanisms underlying tissue tropism are interesting but totally unknown.

Host immune defense and tissue tropism of symbionts. In hemolymph, *Spiroplasma* was abundantly detected, while *Wolbachia* was not (Fig. 2). This pattern is intriguing in that hemolymph is the location of insect innate immune mechanisms, such as the phenol oxidase cascade, inducible antimicrobial peptides, and phagocytic and encapsulating hemocytes (28, 34, 48). Thus, hemolymph probably represents a very hostile environment for microorganisms. It is conceivable that *Spiroplasma* can somehow cope with the hostile environment while *Wolbachia* cannot, resulting in the predominance of *Spiroplasma* in hemolymph. Actually, it was experimentally shown that the male-killing *Spiroplasma* organism was hidden from the host immune defense, although potentially suppressible by it (18). It was also reported that *Wolbachia* infection neither induced nor suppressed the expression of antimicrobial peptides (5), which probably reflects the substantial absence of *Wolbachia* cells in hemolymph.

Effects of coinfection on reproductive phenotypes of *Wolbachia* and *Spiroplasma*. In *D. melanogaster*, the *Spiroplasma* strain NSRO causes almost complete male killing (1), while the *Wolbachia* strain *w*Mel induces weak CI (4). In this study, it was also of interest to determine how these reproductive phenotypes would be affected by the interaction between the coexisting symbionts. The complete male-killing phenotype of *Spiroplasma* was not affected by coinfecting *Wolbachia* organisms (data not shown), in agreement with the density data showing that the population of *Spiroplasma* was not affected by *Wolbachia* (Fig. 1A and B). We could not examine the effect of coinfection on the CI phenotype of *Wolbachia* because few males of the doubly infected strain were available due to male killing.

Coinfection with *Wolbachia* and *Spiroplasma* in natural populations of *D. melanogaster*. In this study, the interactions between *Spiroplasma* and *Wolbachia* coexisting in the same host body were investigated by using artificially generated doubly and singly infected strains of *D. melanogaster*. The question is whether such a *Spiroplasma-Wolbachia* coinfection is actually found in wild *Drosophila* populations. In other words, are the findings in this study applicable to wild *Drosophila* populations? In natural populations of *D. melanogaster*, infection frequencies of *Wolbachia* were reported to range from 18% to 85%, with an average of 34 to 47% (16, 44). In the Bloomington *Drosophila* Stock Center, nearly 30% of the stocks were found to harbor *Wolbachia* (7). On the other hand, in natural populations of *Drosophila* species, infection frequencies of male-killing *Spiroplasma* were reported to be generally low (<5%) (51). In a recent study, 4 of 173 isofemale lines (2.3%) isolated from a Brazilian natural population of *D. melanogaster* were infected with male-killing *Spiroplasma*, and all the infected lines also harbored *Wolbachia* (30). These observations suggest that *Spiroplasma-Wolbachia* coinfection certainly occurs in natural host populations and might affect ecological and evolutionary aspects of the host and the symbionts. In this context, fitness measurements of the doubly infected and singly infected fly strains under different environmental conditions will be of ecological and evolutionary interest.

ACKNOWLEDGMENTS

We thank A. Sugimura, S. Tatsuno, S. Suo, N. Totsuka, and K. Nomura for technical and secretarial assistance and T. Koana and T. Murata for providing fly strains.

This research was supported by the Program for Promotion of Basic Research Activities for Innovation Biosciences (ProBRAIN) of the Bio-Oriented Technology Research Advancement Institution.

REFERENCES

1. **Anbutsu, H., and T. Fukatsu.** 2003. Population dynamics of male-killing and non-male-killing spiroplasmas in *Drosophila melanogaster*. Appl. Environ. Microbiol. **69:**1428–1434.
2. **Anbutsu, H., and T. Fukatsu.** Tissue-specific infection dynamics of male-killing and non-male-killing spiroplasmas in *Drosophila melanogaster*. FEMS Microbiol. Ecol., in press. [Online.] doi:10.1111/j.1574-6941.2006.00087.x.
3. **Bourtzis, K., and T. A. Miller.** 2003. Insect symbiosis. CRC Press, Boca Raton, Fla.
4. **Bourtzis, K., A. Nirgianaki, G. Markakis, and C. Savakis.** 1996. *Wolbachia* infection and cytoplasmic incompatibility in *Drosophila* species. Genetics **144:**1063–1073.
5. **Bourtzis, K., M. M. Pettigrew, and S. L. O'Neill.** 2000. *Wolbachia* neither induces nor suppresses transcripts encoding antimicrobial peptides. Insect Mol. Biol. **9:**635–639.
6. **Buchner, P.** 1965. Endosymbiosis of animals with plant microorganisms. Interscience, New York, N.Y.
7. **Clark, M. E., C. L. Anderson, J. Cande, and T. L. Karr.** 2005. Widespread prevalence of *Wolbachia* in laboratory stocks and the implications for *Drosophila* research. Genetics **170:**1667–1675.
8. **Dobson, S. L., K. Bourtzis, H. R. Braig, B. F. Jones, W. Zhou, F. Rousset, and S. L. O'Neill.** 1999. *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. Insect Biochem. Mol. Biol. **29:**153–160.
9. **Dyer, K. A., M. S. Minhas, and J. Jaenike.** 2005. Expression and modulation of embryonic male-killing in *Drosophila innubila*: opportunities for multilevel selection. Evolution **59:**838–848.
10. **Fukatsu, T.** 1999. Acetone preservation: a practical technique for molecular analysis. Mol. Ecol. **8:**1935–1945.
11. **Fukatsu, T., and H. Ishikawa.** 1993. Occurrence of chaperonin 60 and chaperonin 10 in primary and secondary bacterial symbionts of aphids: implications for the evolution of an endosymbiotic system in aphids. J. Mol. Evol. **36:**568–577.
12. **Fukatsu, T., K. Watanabe, and Y. Sekiguchi.** 1998. Specific detection of intracellular symbiotic bacteria of aphids by oligonucleotide-probed *in situ* hybridization. Appl. Entomol. Zool. **33:**461–472.
13. **Fukatsu, T., T. Tsuchida, N. Nikoh, and R. Koga.** 2001. *Spiroplasma* symbiont of the pea aphid *Acyrthosiphon pisum* (Insecta: Homoptera). Appl. Environ. Microbiol. **67:**1284–1291.
14. **Gepner, J., and T. S. Hays.** 1993. A fertility region on the Y chromosome of *Drosophila melanogaster* encodes a dynein microtubule motor. Proc. Natl. Acad. Sci. USA **90:**11132–11136.
15. **Gilbert, S. F.** 2003. Developmental biology, 7th ed. Sinauer Associates Inc., Sunderland, Mass.
16. **Hoffmann, A. A., D. J. Clancy, and E. Merton.** 1994. Cytoplasmic incompatibility in Australian populations of *Drosophila melanogaster*. Genetics **136:** 993–999.
17. **Hoffmann, A. A., M. Hercus, and H. Dagher.** 1998. Population dynamics of the *Wolbachia* infection causing cytoplasmic incompatibility in *Drosophila melanogaster*. Genetics **148:**221–231.
18. **Hurst, G. D. D., H. Anbutsu, M. Kutsukake, and T. Fukatsu.** 2003. Hidden from the host: *Spiroplasma* bacteria infecting *Drosophila* do not cause an immune response, but are suppressed by ectopic immune activation. Insect Mol. Biol. **12:**93–97.
19. **Ijichi, N., N. Kondo, R. Matsumoto, M. Shimada, H. Ishikawa, and T. Fukatsu.** 2002. Internal spatio-temporal population dynamics of triple infection with *Wolbachia* in *Callosobruchus chinensis* (Insecta: Coleoptera: Bruchidae). Appl. Environ. Microbiol. **68:**4074–4080.
20. **Ikeda, T., H. Ishikawa, and T. Sasaki.** 2003. Regulation of *Wolbachia* density in the Mediterranean flour moth, *Ephestia kuehniella*, and the almond moth, *Cadra cautella*. Zool. Sci. **20:**153–157.
21. **Kamoda, S., S. Masui, H. Ishikawa, and T. Sasaki.** 2000. *Wolbachia* infection and cytoplasmic incompatibility in the cricket *Teleogryllus taiwanemma*. J. Exp. Biol. **203:**2503–2509.
22. **Kikuchi, Y., and T. Fukatsu.** 2003. Diversity of *Wolbachia* endosymbionts in heteropteran bugs. Appl. Environ. Microbiol. **69:**6082–6090.
23. **Koga, R., T. Tsuchida, and T. Fukatsu.** 2003. Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. Proc. R. Soc. Lond. B **270:** 2543–2550.
24. **Komaki, K., and H. Ishikawa.** 1999. Intracellular bacterial symbionts of aphids possess many genomic copies per bacterium. J. Mol. Evol. **48:**717–722.
25. **Komaki, K., and H. Ishikawa.** 2000. Genomic copy number of intracellular bacterial symbionts of aphids varies in response to developmental stage and morph of their host. Insect Biochem. Mol. Biol. **30:**253–258.
26. **Kondo, N., N. Ijichi, M. Shimada, and T. Fukatsu.** 2002. Prevailing triple infection with *Wolbachia* in *Callosobruchus chinensis* (Coleoptera: Bruchidae). Mol. Ecol. **11:**167–180.
27. **Kondo, N., M. Shimada, and T. Fukatsu.** 2005. Infection density of *Wolbachia* endosymbiont affected by co-infection and host genotype. Biol. Lett. **1:**488–491.
28. **Lavine, M. D., and M. R. Strand.** 2002. Insect hemocytes and their role in immunity. Insect Biochem. Mol. Biol. **32:**1295–1309.
29. **Mira, A., and N. A. Moran.** 2002. Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria. Microb. Ecol. **44:**137–143.
30. **Montenegro, H., V. N. Solferini, L. B. Klaczko, and G. D. D. Hurst.** 2005. Male-killing *Spiroplasma* naturally infecting *Drosophila melanogaster*. Insect Mol. Biol. **14:**281–287.
31. **Mouton, L., H. Henri, M. Bouletreau, and F. Vavre.** 2003. Strain-specific regulation of intracellular *Wolbachia* density in multiply infected insects. Mol. Ecol. **12:**3459–3465.
32. **Mouton, L., F. Dedeine, H. Henri, M. Bouletreau, N. Profizi, and F. Vavre.** 2004. Virulence, multiple infections and regulation of symbiotic population in the *Wolbachia-Asobara tabida* symbiosis. Genetics **168:**181–189.
33. **Mouton, L., H. Henri, M. Bouletreau, and F. Vavre.** 2005. Multiple infections and diversity of cytoplasmic incompatibility in a haplodiploid species. Heredity **94:**187–192.
34. **Naitza, S., and P. Ligoxygakis.** 2004. Antimicrobial defences in *Drosophila*: the story so far. Mol. Immunol. **40:**887–896.
35. **Noda, H., Y. Koizumi, Q. Zhang, and K. Deng.** 2001. Infection density of *Wolbachia* and incompatibility level in two planthopper species, *Laodelphax striatellus* and *Sogatella furcifera*. Insect Biochem. Mol. Biol. **31:**727–737.
36. **O'Neill, S. L., A. A. Hoffmann, and J. H. Werren.** 1997. Influential passengers: inherited microorganisms and arthropod reproduction. Oxford University Press, New York, N.Y.
37. **Poulson, D. F., and B. Sakaguchi.** 1961. Nature of "sex-ratio" agent in *Drosophila*. Science **133:**1489–1490.
38. **Poulson, D. F., and B. Sakaguchi.** 1961. Hereditary infections in *Drosophila*. Genetics **46:**890–891.
39. **Riegler, M., M. Sidhu, W. J. Miller, and S. L. O'Neill.** 2005. Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*. Curr. Biol. **15:** 1428–1433.
40. **Rigaud, T., P. S. Pennings, and P. Juchault.** 2001. *Wolbachia* bacteria effects after experimental interspecific transfers in terrestrial isopods. J. Invertebr. Pathol. **77:**251–257.
41. **Russell, J. A., A. Latorre, B. Sabater-Munoz, A. Moya, and N. A. Moran.** 2003. Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. Mol. Ecol. **12:**1061–1075.
42. **Sakaguchi, B., and D. F. Poulson.** 1961. Distribution of "sex-ratio" agent in tissues of *Drosophila willistoni*. Genetics **46:**1665–1676.
43. **Sakurai, M., R. Koga, T. Tsuchida, X.-Y. Meng, and T. Fukatsu.** 2005. *Rickettsia* symbiont in the pea aphid *Acyrthosiphon pisum*: novel cellular tropism, effect on host fitness, and interaction with the essential symbiont *Buchnera*. Appl. Environ. Microbiol. **71:**4069–4075.
44. **Solignac, M., D. Vautrin, and F. Rousset.** 1994. Widespread occurrence of the proteobacteria *Wolbachia* and partial cytoplasmic incompatibility in *Drosophila melanogaster*. C. R. Acad. Sci. (Paris) **317:**461–470.
45. **Stouthamer, R., J. A. Breeuwer, and G. D. D. Hurst.** 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annu. Rev. Microbiol. **53:**71–102.
46. **Thao, M. L., M. A. Clark, L. Baumann, E. B. Brennan, N. A. Moran, and P. Baumann.** 2000. Secondary endosymbionts of psyllids have been acquired multiple times. Curr. Microbiol. **41:**300–304.
47. **Thao, M. L., P. J. Gullan, and P. Baumann.** 2002. Secondary (gamma-*Proteobacteria*) endosymbionts infect the primary (beta-*Proteobacteria*) endosymbionts of mealybugs multiple times and coevolve with their hosts. Appl. Environ. Microbiol. **68:**3190–3197.
48. **Theopold, U., D. Li, M. Fabbri, C. Scherfer, and O. Schmidt.** 2002. The coagulation of insect hemolymph. Cell. Mol. Life Sci. **59:**363–372.
49. **Veneti, Z., M. E. Clark, T. L. Karr, C. Savakis, and K. Bourtzis.** 2004. Heads or tails: host-parasite interactions in the *Drosophila-Wolbachia* system. Appl. Environ. Microbiol. **70:**5366–5372.
50. **Werren, J. H.** 1997. Biology of *Wolbachia*. Annu. Rev. Entomol. **42:**587–607.
51. **Williamson, D. L., and D. F. Poulson.** 1979. Sex ratio organisms (spiroplasmas) of *Drosophila*, p. 175–208. *In* R. F. Whitcomb and J. G. Tully (ed.), The mycoplasmas, vol. 3. Academic Press, New York, N.Y.
52. **Zhou, W., F. Rousset, and S. L. O'Neill.** 1998. Phylogeny and PCR based classification of *Wolbachia* strains using *wsp* gene sequences. Proc. R. Soc. Lond. B **265:**509–515.